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The overall goal of this research project was to systematically investigate a number of the possible ways through which presynaptic modulation might influence the effectiveness ot local synaptic interactions at the mammalian hippocampal mossy fiber synapse. Work conducted during this period of support resulted in the publication of 20 peer-reviewed research articles and 19 abstracts. These funds also supported the research training of 7 doctoral students, 1 masters student, 2 medical students and 1 high school student.

A hippocampal subcellular fraction that is highly enriched in large mossy fiber nerve endings was developed as a model system for investigating the presynaptic modulation of the hippocampal mossy fiber system. The morphological and metabolic properties of this synaptosomal preparation were described in detail and have been independently verified (Ueda et al., 1992, Eur. J. Pharmacol. 219:271-277). We were the first to demonstrate that both glutamate and

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prodynorphin-derived peptides are co-released from these unusually large nerve endings in response to membrane depolarization by calcium-dependent mechanisms. It was demonstrated that distinct types of voltage-gated calcium channels are required for the exocytosis of glutamate and dynorphin peptides. As originally proposed, several specific hypotheses were systematically tested concerning presynaptic receptors and the autoregulation of the hippocampal mossy fiber synapse. Specifically, it was demonstrated that the transmitter(s) released from the mossy fiber terminals may mediate positive or negative feedback control of the mossy fiber synaptic input, under appropriate conditions, by activating presynaptic autoreceptors.

Our studies demonstrated that at least two different types of glutamatergic autoreceptors are present on the mossy fiber terminals; an inhibitory L(+)aminophosphonobutyric acid-sensitive receptor and an excitatory kainate subtype of receptor. Presynaptic facilitory kainate receptors are hypothesized to enhance mossy fiber transmitter release through a mechanism that involves the activation of a guanine nucleotide-binding regulatory protein (G_s) that stimulates adenylyl cyclase and increases the activity of voltage-gated calcium channels. This presynaptic facilitation may contribute to hippocampal neurodegeneration produced by the plant-derived toxins kainate and domoate.

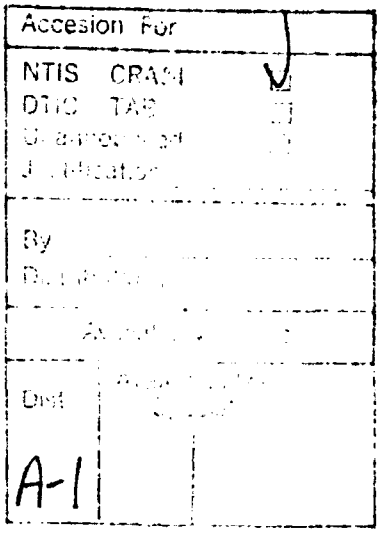
We were also the first to demonstrate that the release of dynorphin from mossy fiber terminals was capable of directly influencing the strength of synaptic transmissioin through the activation of presynaptic inhibitory kappa opioid receptors. Within the past year the physiological significance of this inhibitory control has been independently confirmed (Wagner et al., 1993, Nature 363:451-454). It is our hypothesis that presynaptic kappa opioid receptors may exert an antagonistic influence on mossy fiber transmitter release that may function to limit the overexcitation of hippocampal neurons.

At a behavioral level, we have discovered that kappa opioid agonists act within the hippocampal formation to exert an extremely potent anxiolytic effect, and relatively weak hypotensive effect, in rats. An elevated-plus maze was used to demonstrate that the peripheral injection of these agents produced a dose-dependent anxiolysis that could be completely reversed by the intrahippocampal injection of a selective kappa opioid receptor antagonist.

Finally, we have investigated the role of protein kinase C in the regulation of glutamate and dynorphin release from mossy fiber terminals. Here, it was discovered that the evoked release of glutamate, but not opioid peptides, from isolated mossy fiber terminals was significantly enhanced following the sustained activation of protein kinase C. Although a phorbol ester was used to sustain protein kinase C activation, the selective enhancement of glutamate release persisted for at lease 20 min after the complete removal of the phorbol ester. In striking contrast to the acute effects of phorbol esters, sustained protein kinase C activation lead to an enhancement of glutamate release that was no longer sensitive to inhibitors of this kinase enzyme. Overall, substantial progress has been made from the in vitro analysis of isolated hippocampal mossy fiber synaptosomes. Recent work in this laboratory has resulted in the development of an alternative model systems for use in this project; a primary culture of cortical neurons in which transient transfections can be obtained using plasmid constructions expressing specific PKC isoforms. Through the combined use of these complimentary preparations, both direct and indirect approaches will be employed for the investigation of the relationship between PKC activation, substrate phosphorylation, cytoskeletal reorganization and glutamate exocytosis.

It has been proposed that a controlled and restricted reorganization of the subsynaptic cytoskeleton plays a critical role in plasticity and the maintenance of cell polarity, an inherent feature of cells in the nervous system. However, neuronal processes would atrophy when this same process of cytoskeletal disaggregation (solation) persists beyond

what is required to enhance synaptic strength. Our research has focused on specific molecular components that are sorted and assembled into segregated microdomains surrounding voltage-regulated Ca^{2+} channels on either side of the hippocampal mossy fiber synapse. We postulate that the functional integrity of these microdomains is determined by Ca^{2+} channel activity. In collaboration with Dr. Huntington Potter (Harvard Medical School), we are conducting an experimental investigation of the mechanisms that target specific subsets of mRNA to the dendritic spines of the MF synapses.



PRESYNAPTIC MODULATION OF THE HIPPOCAMPAL MOSSY FIBER SYNAPSE

AFOSR 89-0531

FINAL TECHNICAL REPORT

1. Summary

The overall goal of this research project was to systematically investigate a number of the possible ways through which presynaptic modulation might influence the effectiveness of local synaptic interactions at the mammalian hippocampal mossy fiber synapse. Work conducted during this period of support resulted in the publication of 20 peer-reviewed research articles and 19 abstracts. These funds also supported the research training of 7 doctoral students, 1 masters student, 2 medical students and 1 high school student.

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physiological significance of this inhibitory control has been independently confirmed (Wagner et al., 1993, Nature 363:451-454). It is our hypothesis that presynaptic kappa opioid receptors may exert an antagonistic influence on mossy fiber transmitter release that may function to limit the overexcitation of hippocampal neurons.

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2. Research Objectives

The original research objectives for the funding period 15 September 1989-14 July 1993 were as follows:

a) To "test a number of hypotheses related to the autoregulation of neurotransmitter release from mossy fibers."

b) To "test a number of hypotheses related to the heterosynaptic modulation of neurotransmitter release from mossy fibers."

c) To "test the hypothesis that ecto endopeptidases generate and degrade dynorphin peptides in the mossy fiber synapse."

d) To "conduct pilot experiments to determine whether cholecystokinins are released from isolated mossy fiber nerve endings."

e) To test specific hypotheses related to the presynaptic mechanisms of opioid and acidic amino acid transmitter release from mossy fibers."

3. Status of Research

3.1 To "test a number of hypotheses related to the autoregulation of neurotransmitter release from mossy fibers."

Eight manuscripts were published in which the results of these experiments were discussed in detail (see Publications No. 1, 3, 9, 12, 13, 14, 15, and 19 in section 4.1, below).

A major objective of this study was to determine whether the hippocampal mossy fiber presynaptic terminals represent an anatomical substrate through which kainate-induced neurotoxicity is mediated in the hippocampal CA3 subfield. The impetus for this study comes from the finding that kainate selectively lesions neurons in the CA3 subfield of the hippocampus and that this lesion markedly resembles that associated with prolonged periods of status epilepticus in humans. Kainate has been used extensively in neurobiological research to create an animal model of temporal lobe epilepsy. However, the specific mechanisms by which kainate acts to produce this selective lesion of the hippocampal CA3 subfields remains unknown. Delineation of the presynaptic mechanisms involved in kainate-induced neurotoxicity in animal models may provide crucial information for the development of therapeutic strategies that can be employed in the treatment of temporal lobe epilepsy and other related neurological diseases.

The purpose of our first series of experiments was to critically examine the appropriateness of using isolated mossy fiber nerve endings (synaptosomes) as a model for investigating the presynaptic modulation of glutamate exocytosis from hippocampal mossy fibers. The finding that the incomplete elimination of the dentate granule cells and their mossy fiber axons resulted in a concomitant decrease in the release of both glutamate and dynorphin B confirmed that glutamate exocytosis originates from mossy fiber nerve endings during the depolarization of this subcellular fraction (Conner-Kerr et al., 1992). Therefore, it was concluded that the P_3 subcellular fraction, although not a homogeneous preparation

of mossy fiber synaptosomes, is very highly enriched in the large mossy fiber presynaptic boutons. The results indicated that the continued use of the P_3 fraction for the purpose of my investigations was justified and reasonable. As such, this preparation offered several advantages over conventional hippocampal synaptosomal preparations or the in vitro hippocampal slice.

The second series of experiments were conducted to determine the presynaptic effects of domoate and kainate on glutamate release from hippocampal mossy fiber nerve terminals. The results obtained in this study were consistent with the hypothesis that the neurotoxic effects of these excitatory amino acids in the hippocampal CA3 subfield may be mediated, at least in part, by the presynaptic facilitation of glutamate exocytosis (Gannon and Terrian, 1991; Terrian et al., 1991). The localization of a presynaptic site of action for kainate and domoate also provided an explanation for the observation that an intact hippocampal mossy fiber pathway is required for the expression of kainate- and domoate-induced neurotoxicity in the hippocampal CA3 subfields. The specific findings of this study included the following:

First, both domoate and kainate significantly potentiated the depolarization-induced, Ca^{2+}-dependent release of endogenous glutamate from hippocampal mossy fiber synaptosomes (Gannon and Terrian, 1991b; Terrian et al., 1991a). In addition, domoate was found to be three times more potent than kainate in augmenting the depolarization-evoked release of endogenous glutamate. This finding is in agreement with electrophysiological studies that have shown domoate to be a more potent excitant of the hippocampal CA3 neurons than kainate.

Second, the facilitatory actions of both domoate and kainate were completely blocked by the non-NMDA antagonist CNQX (Terrian et al., 1991). This finding demonstrated that domoate and kainate acted through a non-N-methyl-D-aspartate subtype of excitatory amino acid receptor.

Third, evidence provided by this study also indicated that domoate and kainate may interact with a common molecular component of the presynaptic membrane. When combined, the effects of domoate and kainate on glutamate release were not additive and did not produce a significantly greater potentiation of endogenous glutamate release than kainate alone (Terrian et al., 1991a).

Fourth, neither domoate nor kainate had an effect on the spontaneous efflux of endogenous glutamate from hippocampal mossy fiber synaptosomes (Terrian et al., 1991a). This finding suggests that the modulatory actions of domoate and kainate are dependent on membrane depolarization.

Fifth, since membrane depolarization was required for domoate and kainate-induced potentiation of endogenous glutamate release, the effects of these two excitotoxins were tested on the depolarization-induced rise in $[Ca^{2+}]_i$. Preliminary findings indicated that domoate and kainate enhanced the extent to which depolarization increased the level of intrasynaptosomal calcium (Terrian et al., 1991a). However, the effects of domoate and kainate on the depolarization-induced rise in intraterminal free calcium could not be fully explored because higher concentrations (> 100 μM) of these compounds interfered with the emission of fluorescence by the fura-2 dye.

A second major objective of our experiments was to determine whether dynorphin opioid peptides interact with an autoreceptor to modulate the release of neurotransmitters from the hippocampal mossy fiber terminals. Opioid agonists specific for the μ, δ and κ opioid receptor subtypes were tested for their ability to modulate potassium-evoked release of glutamate and dynorphin B-like immunoreactivity from guinea pig hippocampal mossy fiber synaptosomes. The κ opioid agonists U-62,066E and (-)ethylketocyclazocine, but not the μ agonist [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO) nor the δ agonist [D-Pen^{2,5}]enkephalin (DPDE), inhibited the potassium-evoked release of L-glutamate and dynorphin B-like immunoreactivity. U-62,066E, but not DAGO or DPDE, also inhibited the potassium-evoked rise in mossy fiber synaptosomal cytosolic Ca^{2+} levels, indicating a possible mechanism for κ agonist inhibition of transmitter release. DAGO and DPDE were found to be without any effect on cytosolic Ca^{2+} levels or transmitter release in this preparation. The U-62,066E inhibition of the potassium-evoked rise in synaptosomal cytosolic Ca^{2+} levels was partially attenuated by the opioid antagonist quadazocine and insensitive to the δ-opioid specific antagonist ICI 174,864 and the μ opioid-preferring antagonists naloxone and naltrexone. Quadazocine also reversed U-62,066E inhibition of the potassium-evoked release of L-glutamate, but not dynorphin B-like immunoreactivity. These results suggest that κ opioid agonists inhibit transmitter release from mossy fiber terminals through both κ opioid and non-κ opioid receptor mediated mechanisms.

3.2 To "test a number of hypotheses related to the heterosynaptic modulation of neurotransmitter release from mossy fibers."

Five manuscripts were published in which the results of these experiments were discussed in detail (see Publications No. 4, 6, 10, and 16 in section 4.1, below).

The presynaptic effects of muscarine on hippocampal mossy fiber synaptic transmission were examined using a subcellular fraction enriched in isolated mossy fiber synaptosomes. Muscarine significantly enhanced the K^+-evoked release of endogenous glutamate but not dynorphin from superfused guinea pig hippocampal mossy fiber synaptosomes. The presynaptic facilitation of glutamate release by muscarine was dose-dependent and was antagonized by the prior application of atropine. The effects of a variety of alternative cholinergic agonists on the depolarization-induced rise in $[Ca^{2+}]_i$ were also tested using the Ca^{2+} indicator dye, fura 2. Neither muscarinic nor nicotinic agonists produced a change in the extent to which membrane depolarization augmented the availability of $[Ca^{2+}]_i$ in mossy fiber synaptosomes. These results are consistent with the hypothesis that extrinsic septal cholinergic inputs to the hippocampal CA3 region may generate synchronized firing in pyramidal neurons through a mechanism which involves the presynaptic facilitation of the excitatory mossy fiber synaptic input.

3.3 To "test the hypothesis that ecto-endopeptidases generate and degrade dynorphin peptides in the mossy fiber synapse."

We were unable to directly test this hypothesis and no publishable data were obtained as a result of our repeated efforts to address this issue in a meaningful manner.

3.4 To "conduct pilot experiments to determine whether cholecystokinins are released from isolated mossy fiber nerve endings."

Before we could initiate these experiments Verhage et al. (1991, Neuron 6:517-524) had already published the results of their study in which it was demonstrated that hippocampal synaptosomes do indeed release cholecystokinin-like immunoreactivity in response to membrane depolarization. We have not attempted to repeat this study, since the results reported by Verhage et al. (1991) seem reliable. However, we have extended the investigation of neuropeptide release and its differential regulation by demonstrating that methionine-enkephalin is also released from hippocampal mossy fiber synaptosomes. Thus, a variety of neuropeptides may be co-released and potentially play a role in the autoregulation of glutamate release from these large nerve endings.

3.5 To test specific hypotheses related to the presynaptic mechanisms of opioid and acidic amino acid transmitter release from mossy fibers."

Eight manuscripts were published in which the results of these experiments were discussed in detail (see Publications No. 2, 5, 7, 8, 11, 17, 18, and 20 Section 4.1, below).

A systematic investigation was conducted to identify the second messenger system(s) which mediate the kainate-induced potentiation of Glu release. Cyclic adenosine monophosphate (cAMP)-dependent, guanine nucleotide regulatory binding-protein and calcium/calmodulin-dependent protein kinase II (Ca_{2+}/CaM-dependent PK II) systems were studied.

The purpose of our first study was to determine if cAMP-dependent processes modulated the facilitatory actions of kainate on glutamate release. A basis for this study was provided by the finding that ionotropic glutamate receptors have a large intracellular loop that connects transmembrane segments 3 and 4 of the receptor protein which contains consensus phosphorylation sites for many protein kinases. In addition, it has been reported that incubation of cultured hippocampal neurons with cell permeant cAMP analogues, or the catalytic subunit of cAMP-dependent protein kinase (PKA), results in an enhancement of kainate-induced currents. Therefore, the effects of cAMP-dependent processes on the kainate-induced potentiation of glutamate release from hippocampal mossy fiber nerve terminals were examined. However, no support for the regulation of the kainate subtype

of glutamate receptor by the cAMP-dependent protein kinase system was found. Superfusion of mossy fiber synaptosomes with a cell permeant cAMP analogue, Sp-cAMP, neither augmented nor reduced the facilitatory actions of kainate on the depolarization-evoked release of endogenous glutamate. In addition, no effect of cAMP-dependent processes were detected on the K^+-evoked release of glutamate. The possibility remains, however, that other protein kinase systems endogenous to mossy fiber nerve terminals might regulate the activity of the kainate receptor. For example, we have demonstrated that protein kinase C augments the depolarization-induced release of glutamate from mossy fiber synaptosomes (Terrian et al., 1991). Therefore, the next logical step would be to investigate the effects of protein kinase C-dependent processes on the kainate-induced potentiation of glutamate release.

In a separate series of experiments we attempted to determine whether G_S-proteins couple kainate receptors to an effector mechanism that is capable of facilitating Glu exocytosis. Separation of hippocampal mossy fiber synaptosomal membranes that had been treated with cholera toxin and [^{32}P]NAD by SDS-PAGE revealed the presence of G_S proteins in hippocampal mossy fiber nerve terminal membranes. The functional disengagement of G_S proteins from membrane receptors by cholera toxin-induced ADP-ribosylation had no effect on the evoked release of glutamate or on the ability of kainate to potentiate the evoked release of glutamate. This study does not provide evidence to support a role for G_S proteins in transducing kainate receptor activation into an intracellular signal. However, the involvement of G_S proteins can not be entirely ruled out since only partial ADP-ribosylation of G_S proteins could be achieved in intact mossy fiber synaptosomes. As a result of this partial ADP-ribosylation, a percentage of the G_S proteins present in mossy fiber synaptosomes are, presumably, still capable of transducing KA-generated signals into intracellular messages.

A third study was conducted to determine if the facilitatory effect of kainate on evoked glutamate release was mediated by Ca^{2+}/CaM-dependent PK II. Recent studies have suggested a role for this kinase in the molecular mechanisms that mediate synaptic vesicle exocytosis. In the presence of barium, an ion that does not support the activation of Ca^{2+}/CaM-dependent PK II, no effect could be detected on the kainate-induced potentiation of evoked glutamate release. The inhibition of Ca^{2+}/CaM-dependent PK II also did not suppress the K^+-evoked release of glutamate. In fact, barium was found to be a more effective secretagogue than calcium in hippocampal mossy fiber synaptosomes. These findings indicate that activation of the Ca^{2+}/CaM-dependent PK II system is not required for the kainate-induced potentiation of evoked glutamate release from hippocampal mossy fiber nerve terminals. In addition, our findings that the K^+-evoked release of glutamate was enhanced by the Ca^{2+}/CaM PK II inhibitor, KN-62, and the substitution of barium for calcium suggests that the phosphotransferase activity mediated by this kinase may actually suppress, rather than facilitate, glutamate exocytosis.

The inclusion of tetrodotoxin in the superfusion medium, to prevent barium from depolarizing the synaptosomal plasma membrane, also did not have any effect on kainate-

induced potentiation of glutamate release. This finding indicates that the effects of kainate are not due to the activation of voltage-regulated sodium channels. Moreover, our findings are consistent with the observation that tetrodotoxin did not block kainate-induced potentiation of glutamate release from depolarized cerebellar slices.

The preliminary results from our fura-2 experiments indicated that the facilitatory effects of domoate and kainate on evoked glutamate release might be mediated via an increase in the levels of intracellular calcium. However, the results of our most recent study (Conner Kerr and Terrian, 1993) conclusively demonstrate that calcium entry is not required for the kainate-induced facilitation of glutamate release. By excluding this possibility, it is considered that this work will have a significant and enduring impact on future investigations of excitotoxicity.

In conclusion, evidence presented here and in earlier reports, suggests that the susceptibility of the hippocampal CA3 cells to kainate may result from the localization of kainate receptors on hippocampal mossy fiber terminals. Therefore, kainate-induced neurotoxicity in the hippocampal CA3 subfield appears to be mediated, at least in part, by presynaptic mechanisms. The identification of a specific anatomical substrate for kainate will undoubtedly facilitate future studies of the molecular mechanisms involved in the kainate-induced potentiation of glutamate exocytosis.

Our most recent studies have focused on the effects of protein kinase C-dependent phosphotransferase activity on the co-release of dynorphin and glutamate from hippocampal mossy fiber nerve endings. This has proven to be a very productive field of investigation in this laboratory during the past year. In our most recent study we have investigated the delayed and persistent effects of 4β-phorbol 12,13-dibutyrate (PDBu) on the K^+-evoked release of endogenous glutamate and dynorphin B-like immunoreactivity from hippocampal mossy fiber synaptosomes. It was demonstrated that the α, β, γ, ε, and ζ isoforms of protein kinase C are present in these nerve endings. The K^+-evoked release of glutamate was found to be selectively enhanced when mossy fiber-enriched synaptosomes were preincubated with PDBu for 15 min and extensively washed with a PDBu-free medium. In subsequent studies we have routinely incubated intact synaptosomes for 15 min with PDBu and then superfused with a PDBu-free medium. This has allowed us to examine the persistent, rather than acute, effects of PKC activation in the absence of PDBu. The amount of glutamate released, during the slow phase, was increased two-fold by this pretreatment. Down-regulation of PKC, during the additional 32 min required to washout PDBu, did not account for this enhancement of release. Three independent criteria support this conclusion. First, western blot analyses were performed to confirm that the total synaptosomal content of PKC α, β, γ, ε and ζ had not decreased *prior to* the enhancement of glutamate release. Second, PKC-dependent phosphotransferase activity, measured using two different exogenous substrates, reported no decrease in PKC activity. Third, during more prolonged (45-180 min) pretreatments, there was no correlation between the extent to which PDBu enhanced glutamate release and the pattern of down-regulation for the 5 isoforms examined. Using TPA and phosphatidylserine, we

also identified the presence of a number of putative endogenous PKC substrates in a synaptosomal cytosolic fraction. However, PDBu only changed the phosphorylation of two substrates (M_r 50 kDa and 85 kDa) in polarized, intact, synaptosomes. After 45 min, PDBu dramatically reduced the basal level of P85 phosphorylation. In contrast, the level of phosphorylation was increased at later times for other endogenous substrates. P85 band was also prevalent in the solubilized particulate fraction of controls, but could no longer be detected in this fraction after 45 min with PDBu. Finally, it was demonstrated that the glutamate release apparatus had become desensitized following the removal of PDBu, since the reintroduction of PDBu into the superfusion medium did not further enhance the K^+-evoked release of this amino acid.

During continuous depolarization in the presence of 0.9 mM $CaCl_2$, an identical pretreatment with 1 μM PDBu resulted in a far more substantial increase in the final extent of glutamate release, but did not extend the time over which this release could be sustained. Thus, PDBu increased the size of the total pool of glutamate that was available for release, but apparently did not disengage those factors which act to terminate secretion.

The magnitude of PDBu-enhanced glutamate release is dependent on both the dose of PDBu and duration of preincubation. At a concentration of 1 μM PDBu, a 5 min preincubation is not "adequate" to reliably enhance glutamate release following the 36 min washout period. Using the same protocol, we have found that a 10 min exposure to 1 μM PDBu is at least as effective as a 15-45 min exposure to 10 μM PDBu. Extending the washout period (superfusion) to 50 min did not reduce the effect of PDBu (data not shown). Thus, the minimal conditions of PDBu exposure that appear to be required for the reliable enhancement of glutamate release have been delineated (i.e.; 10 min with 1.0 μM PDBu).

The route by which Ca^{2+} enters the nerve terminal is an important determinant of the extent of glutamate release. Our results confirm that the Ca^{2+} ionophore, ionomycin (ION), is more effective than depolarizing agents in increasing the overall level of $[Ca^{2+}]_i$, but is an ineffective stimulus of glutamate release (4-AP, 4-aminopyridine). Because depolarizing agents cause Ca^{2+} to enter at the release sites, the extent of glutamate release is disproportionately increased when compared to the release induced by ionomycin. These findings lead to the prediction that a physiologically relevant increase in "priming efficiency" should preferentially enhance the extent of *depolarization*-induced glutamate release. The testing of this prediction represented the departure point for a preliminary study we have recently conducted. PDBu (1 μM) selectively enhanced the depolarization-induced (35 mM KCl and 3 mM 4-AP) release of glutamate, but had no effect on ionomycin-induced release. This result stands in striking contrast to the finding that the introduction of autoactivated CaM kinase II into synaptosomes increased both the ION (5 μM) and K^+-evoked release of glutamate to the same extent. Thus, PDBu appears to selectively prime the glutamate release apparatus that is preferentially responsive to localized Ca^{2+} entry in intact synaptosomes.

The PDBu-induced enhancement of glutamate release was completely blocked by the A_f destabilizing agent, cytochalasin D. This fungal metabolite permeates cell membranes and causes actin filament networks to change from an isotropic network to focal accumulations by binding to the barbed end of actin filaments. We have found that cytochalasin D significantly increased the K^+-evoked, but not basal, release of glutamate and 'occluded' PDBu-enhanced glutamate release.

The K^+-induced rise in bulk synaptosomal $[Ca^{2+}]_i$, reported by fura 2-AM, is not increased by the pretreatment of mossy fiber-enriched synaptosomes with 10 μM PDBu for 45 min. These responses were measured after washing synaptosomes to remove PDBu. Lower concentrations of PDBu ar ' shorter incubation times were also without effect (data not shown). Although PDBu did not alter the rapid phase of Ca^{2+} entry, this does not prove that the *initial* localized entry of Ca^{2+} was not significantly increased. However, this transient phase of Ca^{2+} entry is thought to trigger the release of glutamate from a small (10-20%) releasable pool. It is unlikely that the facilitation of Ca^{2+} channel activity could entirely account for the two-fold increase in glutamate release we have observed.

A plausible explanation for our preliminary results might be that the PDBu-induced phosphorylation of CaM-binding substrates of PKC could enhance the release of glutamate through the phosphorylation-induced liberation of free calmodulin (CaM) and activation of CaM kinase II. To examine this possibility, mossy fiber-enriched synaptosomes were preincubated in the presence of either 1 μM 4-α phorbol or PDBu for 45 min, washed four times with a phorbol-free medium, and the level of autonomous CaM kinase phosphotransferase activity was assayed *in vitro*, using the CaM kinase-selective substrate, autocamtide-2. PDBu did not alter the basal level of autonomous CaM kinase activity or the activation of CaM kinase during the localized (35 mM KCl) and delocalized (1 μM ION) entry of Ca^{2+}. Both of the latter stimuli produce a submaximal rise in Ca^{2+} and, therefore, any priming of CaM kinase by the liberation of free CaM should have been detected. In addition, we have shown that K^+-evoked release was more than doubled when $BaCl_2$ was used in equimolar substitution for $CaCl_2$ in the depolarizing medium. $BaCl_2$ by itself did not increase the spontaneous efflux of glutamate because 0.1 μM tetrodotoxin was included in the superfusion medium. It is well established that Ba^{2+} supports PKC-dependent phosphotransferase activity, but does not substitute for Ca^{2+} in the activation of CaM kinase II or phosphorylation of synapsin I. These results have been submitted for publication and imply that PKC-CaM kinase II 'cross-talk' cannot entirely account for PDBu-induced enhancement of glutamate release.

In sum, our experiments indicate that the stabilized form of PDBu-enhanced glutamate release, following removal of PDBu, selectively increased the pool of glutamate that was available for slow release, from hippocampal mossy fiber nerve endings, in response to localized Ca^{2+} entry. While an organized actin network appeared to be required for the PDBu-induced enhancement of glutamate release, once established, this release apparatus remained "primed" by a process that is: 1) preceded by a 500% increase in MBP_{4-14} phosphorylation, 2) no longer dependent on continued PKC phosphotransferase

or pp-1/2A activity, 4) associated with a decreased basal level of P85 kDa phosphorylation, and 5) cannot be attributed to the down-regulation of endogenous PKC isoforms or 6) the enhancement of autonomous CaM kinase activity.

4. Publications

4.1 Full papers and review articles:

1. Gannon, RL, Baty, LT, TERRIAN, DM: L(+)-2-amino-4-phosphonobutyrate inhibits the release of both glutamate and dynorphin from guinea pig but not rat hippocampal mossy fiber synaptosomes. Brain Res. 1989; 495: 151-155.

2. TERRIAN, DM, Gannon, RL, Damron, DS, Dorman, RV: Effects of calcium antagonists on the evoked release of dynorphin A(1-8) and availability of intraterminal calcium in rat hippocampal mossy fiber synaptosomes. Neurosci. Lett. 1989; 106: 322-327.

3. Gannon, RL, TERRIAN, DM: BOAA selectively enhances L-glutamate release from guinea pig hippocampal mossy fiber synaptosomes. Neurosci. Lett. 1989; 107: 289-294.

4. Dorman, RV, TERRIAN, DM: The role of arachidonic acid and prostaglandins in the neurotransmitter release from isolated cerebellar glomeruli. NYAS 1989; 559: 438-440.

5. TERRIAN, DM, Gannon, RL, Rea, MA: Glutamate is the endogenous amino acid selectively released by rat hippocampal mossy fiber synaptosomes concomitant with prodynorphin-derived peptides. Neurochem. Res. 1990; 15: 1-5.

6. Freeman, E, TERRIAN, DM, Dorman, RV: Presynaptic facilitation of glutamate release from isolated hippocampal mossy fiber nerve endings by arachidonic acid. Neurochem. Res. 1990; 15: 749-756.

7. TERRIAN, DM, Dorman, RV, Gannon, RL: Characterization of the presynaptic calcium channels involved in glutamate exocytosis from rat hippocampal mossy fiber synaptosomes. Neurosci. Lett., 1990; 119: 211-214.

8. TERRIAN, DM, Dorman, RV, Damron, DS, Gannon, RL: Displacement of endogenous glutamate with D-aspartate: an effective strategy for reducing the calcium-

independent component of glutamate release from synaptosomes. Neurochem. Res., 1991; 16: 35-41.

9. Gannon, RL, TERRIAN, DM: Presynaptic modulation of glutamate and dynorphin release by excitatory amino acids in the guinea pig hippocampus. Neuroscience, 1991; 41: 401-410.

10. Freeman, EJ, Damron, DM, TERRIAN, DM, Dorman, RV: 12-Lipoxygenase products attenuate the glutamate release and calcium accumulation evoked by depolarization of hippocampal mossy fiber nerve endings. J. Neurochem., 1991; 56: 1079-1082.

11. TERRIAN, DM, Ways, DK, Gannon, RL: A presynaptic role for protein kinase C in hippocampal mossy fiber synaptic transmission. Hippocampus, 1991; 1: 303-314.

12. Gannon, RL, TERRIAN, DM: U-50,488H inhibits dynorphin and glutamate release from guinea pig hippocampal mossy fiber terminals. Brain Res., 1991; 548: 242-247.

13. TERRIAN, DM, Privette, TH, Conner-Kerr, T, Gannon, RL: Domoic acid enhances the K^+-evoked release of glutamate from guinea pig hippocampal mossy fiber synaptosomes. Brain Res., 1991; 551: 303-307.

14. Gannon, RL, TERRIAN, DM: Kappa opioid agonists inhibit transmitter release from guinea pig hippocampal mossy fiber synaptosomes. Neurochem. Res., 1992; 17: 741-747.

15. Simpson, J., Gannon, RL, McGinty, JF, TERRIAN, DM: Kainic acid depresses the ex vivo release of dynorphin B and glutamate from rat hippocampal mossy fiber synaptosomes. Neurosci. Lett., 1992; 137: 149-153.

16. Loewen, JJ, Peters, RI, TERRIAN, DM: Adenosine modulation of dynorphin B release by hippocampal synaptosomes. Brain Res., 1992; 577: 318-320.

17. TERRIAN, DM, Ways, DK, Gannon, RL, Zetts, DA: Transduction of a protein kinase C-generated signal into the long-lasting potentiation of glutamate release. Hippocampus, 1993; 3: 205-220.

18. Conner-Kerr, TA, Simmons, D, Peterson, GM, TERRIAN, DM: Evidence for the co-release of dynorphin and glutamate from rat hippocampal mossy fiber terminals. J. Neurochem., 1993; 61: 627-636.

19. Conner-Kerr, TA, TERRIAN, DM: Presynaptic inhibition of glutamate release from hippocampal mossy fiber synaptosomes: a potential mechanism for the anticonvulsant effects of U-54494A. Brain Res. Bull., 1993; 31:573-580.

20. Chicurel, ME, TERRIAN, DM, Potter, H: mRNA at the synapse: analysis of a synaptosomal preparation enriched in hippocampal dendritic spines. J. Neurosci., In Press.

4.2 Refereed Abstracts:

1. Irwin, LN, Gannon, RL, TERRIAN, DM: Depolarization displaces synaptosomal gangliosides. Trans. Am. Soc. Neurochem 1990; 21:311.

2. TERRIAN, DM, Ways, DK, Gannon, RL: Evidence for a presynaptic role of protein kinase C in hippocampal mossy fiber synaptic transmission. Trans. Soc. Neurosci. 1990; 16(1):144.

3. Gannon, RL, TERRIAN, DM: Presynaptic inhibition of hippocampal mossy fiber synaptic transmission by kappa opioids. Trans. Soc. Neurosci. 1990; 16(1):367.

4. Chicurel, ME, TERRIAN, DM, Potter, H: Subcellular localization of mRNA: isolation and characterization of mRNA from an enriched preparation of hippocampal dendritic spines. Trans. Soc. Neurosci. 1990; 16(1):344.

5. Damron, DS, Freeman, EJ, TERRIAN, DM, Dorman, RV: Arachidonic acid-induced calcium mobilization in hippocampal mossy fiber synaptosomes. Trans. Soc. Neurosci. 1990; 16(1):166.

6. Freeman, EJ, Damron, DS, TERRIAN, DM, Dorman, RV: Inhibition of glutamate release from hippocampal mossy fiber synaptosomes by 12-HETE. Trans. Soc. Neurosci. 1990; 16(2):967.

7. Privette, TH, TERRIAN, DM, Zetts, DA, Dorman, RV, and Gannon, RL: Kappa opioid autoregulation of the guinea pig hippocampal mossy fiber synaptosomes. Trans. Am. Soc. Neurochem. 1991; 22(1):221.

8. Conner-Kerr, TA, Gannon, RL, Privette, TH, Patel, MH, and TERRIAN, DM: Domoic acid enhances the release of hippocampal mossy fiber neurotransmitters. Trans. Am. Soc. Neurochem. 1991; 22(1):238.

9 Dorman, RV, Damron, DS, Freeman, EJ, and TERRIAN, DM: Modulation of glutamate release from hippocampal mossy fiber nerve endings by arachidonic acid and eicosanoids. Trans. Intl. Soc. Neurochem. Satellite Meeting on the Neurobiology of Essential Fatty Acids. Cairns, Australia 1991.

10. Simpson, JN, Gannon, RL, McGinty, JF, and TERRIAN, DM: Kainic acid causes a dissociation between the steady-state concentration and the KCl-evoked release of dynorphin B and glutamate from rat hippocampal mossy fiber synaptosomes. Trans. Soc. Neurosci. 1991; 17(1):412.

11. Chicurel, ME, TERRIAN, DM, Harris, KM, and Potter, H: mRNA at the synapse: analysis of a preparation enriched in hippocampal dendritic spine mRNA. Trans. Soc. Neurosci. 1991; 17(1):379.

12. Conner-Kerr, TA, Simmons, DR, Peterson, GM, Zetts, DA, and TERRIAN, DM: Evidence for co-release of dynorphin and glutamate from rat hippocampal mossy fiber terminals. Trans. Am. Soc. Neurochem. 1992; 23:222.

13. TERRIAN, DM, Ways, DK, Dorman, RV, and Zetts, DA: Presynaptic facilitation of glutamate exocytosis may be sustained by activation of a protein kinase cascade. Trans. Soc. Neurosci. 1992; 18:751.

14. Chicurel, ME, DeFranco, C, TERRIAN, DM, and Potter, H: Localization of RNA at synapses: identification of a synaptosomal GAP-43 RNA-binding protein. Trans. Soc. Neurosci. 1992; 18:787.

15. Zetts, DA, Manring, CM, TERRIAN, DM: Priming effect of phorbol esters on evoked glutamate exocytosis. Trans. Am. Soc. Neurochem. 1993; 24:174.

16. Privette, TH and TERRIAN, DM: Reversal of colchicine-induced hyperlocomotion by the kappa opioid agonist U-50,488H. Trans. Am. Soc. Neurochem. 1993; 24:101.

17. Privette, TH, Wang, Q, Patel, M, TERRIAN, DM, and Ingenito, AJ: Possible role for hippocampal dynorphins in SHR hypertension. Trans. Am. Soc. Hypertension. 1993; 6:72A.

18. TERRIAN, DM, Manring, CM, Ways, DK, and Zetts, DA: Priming of glutamate release persists following removal of phorbol ester and is calmodulin-independent. Trans. Soc. Neurosci. 1993; 19:1517.

19. Privette, TH and TERRIAN, DM: Anxiolytic effects and a putative site of action for the kappa opioid agonist U-50,488H. Trans. Soc. Neurosci. 1993; 19:in press.

5. Professional Personnel Associated With the Research Project

David M. Terrian, Ph.D. - Principal Investigator
Robert L. Gannon, Ph.D. - Co-Investigator
Robert V. Dorman, Ph.D. - Collaborator
Ralph I. Peters, Ph.D. - Collaborator
Gary M. Peterson, Ph.D. - Collaborator
Huntington Potter, Ph.D. - Collaborator
D. Kirk Ways, Ph.D., M.D. - Collaborator
Debbie A. Zetts, B.S. - Research Technician III
Meena H. Patel, B.S. - Research Technician II and Medical Student
Christine M. Manring, B.S. - Research Technician II
Marina E. Chicurel, B.S. - Graduate Student
Teresa A. Conner-Kerr, Ph.D. - Graduate Student
Derek M. Damron, Ph.D. - Graduate Student
Earnest J. Freeman, Ph.D. - Graduate Student
Jeffrey J. Loewen, B.S. - Graduate Student
Thomas H. Privette, B.S. - Graduate Student
Jeffrey N. Simpson, B.S. - Graduate Student
Duncan R. Simmons, B.S. - Medical Student
Greck S. Cannon - High School Student

6. Interactions (9/12/92 to 7/15/93)

9/12/92 Invited Lecture, ECU Department of Physiology.

10/25/92 Society for Neuroscience, Anaheim, CA.

11/12/92 Panelist, National Science Foundation, Washington, D.C.

12/8/92 NASA Space Physiology and Countermeasures Peer Review Panel.

3/21/93 American Society of Neurochemistry, Richmond, VA.

4/7/93 Panelist, National Science Foundation, Washington, D.C.

6/2/93 NASA Space Physiology and Countermeasures Peer Review Panel.

6/12/93 Invited Lecture, ECU Department of Anatomy & Cell Biology.

7. New Discoveries, Inventions, or Patent Applications

Patent Application Serial Number 08/014,776: "Method of Treating Anxiety-Related Disorders". Co-inventors David M. Terrian and Thomas H. Privette.